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Microarray analyses in bladder cancer cells: Inhibition of hTERT expression down-regulates EGFR

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The human telomerase reverse transcriptase (hTERT) contributes to the immortal phenotype of the majority of cancers. Targeting hTERT by transfection with antisense oligonucleotides (AS-ODNs) induced immediate growth inhibition in human bladder cancer (BCa) cells. The molecular basis of the antiproliferative capacity of hTERT AS-ODNs was investigated by oligonucleotide microarray analyses and was compared to effects caused by siRNA-mediated knock-down of hTERT in EJ28 BCa cells. Two different AS-ODNs—both down-regulated the expression of hTERT—changed the expression of different genes mainly involved in stress response (including *EGR1*, *ATF3* and *GDF15*), but without an association to telomerase function. This indicates that the immediate growth inhibition was caused, at least in part, by off-target effects. In comparison to that the blockade of the expression of hTERT using 2 different siRNAs was accompanied by the down-regulation of the oncogenes FOS-like antigen 1 (*FOSL1*) and epidermal growth factor receptor (*EGFR*), known to be overexpressed in BCa. We show here for the first time that repression of the hTERT transcript number decreased the expression of EGFR both at the mRNA and protein levels, suggesting a potential new function of hTERT in the regulation of EGFR-stimulated proliferation. Furthermore, the suppression of hTERT by siRNAs caused an enhancement of the antiproliferative capacity of the chemotherapeutics mitomycin C and cisplatin. The results presented herein may support the hypothesis that hTERT promotes the growth of tumor cells by mechanisms independent from telomere lengthening. The detailed clarification of these processes will shed light on the question, whether telomerase inhibitors might constitute suitable anticancer tools.
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Key words: antisense; bladder cancer; EGFR; hTERT; oligonucleotide microarrays; siRNA

The human telomerase reverse transcriptase (hTERT) is the catalytically active component of the telomerase complex.[1] hTERT catalyzes the telomere elongation and associates with telomeres, leading to increased genomic stability and enhanced DNA-repair.[2] Its expression correlates with telomerase activity and is restricted to germ cells, stem cells and to more than 90% of human cancers, whereas most normal human somatic cells have no hTERT expression.[3] The parallel transfer of the *HRAS* oncogene and the SV40 large T antigen together with hTERT into human somatic cells caused the malignant transformation of these cells independent of hTERT's function in telomere elongation.[4,5] Furthermore, the ectopic expression of hTERT in human mammary epithelial cells induced mitogenic genes, such as epidermal growth factor receptor (*EGFR*) and basic fibroblast growth factor (*FGF2*).[6] The close association of hTERT with the tumorigenic process supports the use of hTERT as a specific antitumor target.

Different inhibition approaches were described to interfere with the function of hTERT, whereby—in some of the cases—a lag phase between treatment and effect on cell growth was observed, caused by the time needed for telomere attrition to a critical length. The treatment with a small molecular inhibitor or by the use of a dominant negative mutant of hTERT was effective after overcoming this lag phase.[7,8] In contrast, hTERT can impair cell growth immediately and independent from its function in telomere lengthening.[2,5,9] An immediate proliferation stop was initiated by targeting the hTERT mRNA with ribozymes or antisense oligonucleotides (AS-ODNs).[10–12]

Different AS-ODNs targeting genes overexpressed in tumors were already investigated in clinical studies.[13] More recently, the use of small interfering RNAs (siRNAs) has been described to specifically knock-down selected genes.[14] The decision, which of these techniques is favorable, depends on the appropriate context. The pharmacokinetical properties of AS-ODNs in the field of anticancer therapies are well-known from various animal models and clinical studies. The applicability of siRNAs *in vivo* remains to be evaluated in more detail.

In previous studies, we reported the AS-ODN-mediated knock-down of the hTERT mRNA expression in bladder cancer (BCa) cell lines, leading to an immediate suppression of the growth of these cells.[11,15] However, a critical point in the usage of AS-ODNs is the appearance of off-target effects; nonspecific effects, which are different from the effect on the target mRNA. The aim of the present study was to characterize genome-wide expression profiles of the BCa cell line EJ28 after transfection with 2 hTERT AS-ODNs (ASt2206, ASt2331) as the molecular basis of their growth suppressing function. Furthermore, 2 siRNAs (si-hTERT1 and si-hTERT2) were applied as an alternative method to reduce the hTERT expression, and the resulting expression profiles were analyzed by oligonucleotide microarrays. To differ between specific effects of the hTERT targeting constructs and effects caused by a general AS-ODN-mediated or siRNA-mediated mechanism, AS-ODNs and siRNAs directed at the targets survivin and vascular endothelial growth factor (VEGF) were used. The experiments focused on the target-specificities of 2 different strategies to knock-down hTERT and on the characterization of genes involved in regulatory processes associated with hTERT.

Material and methods

Cell culture and transfection

The human BCa cell line EJ28 was cultivated as described previously.[11] The cells were transiently transfected with ODNs (Invitrogen, Karlsruhe, Germany) and siRNAs (Qiagen, Hilden, Germany) (Table I) at 250 nM complexed using Lipofectin (LF) (Invitrogen) at a LF-nucleic acid-ratio of 3:1 (w/w) or DOTAP (Roche, Mannheim, Germany) at a DOTAP-nucleic acid-ratio of 4:1 (w/w). The siRNAs were designed by a specific algorithm (www.qiagen.com). The cells including those floating in the supernatant were harvested and pooled at different time points. Aliquots for RNA extraction and Western blotting were collected in parallel. T1

Combination of hTERT siRNAs and chemotherapeutic agents

Cisplatin (CDDP) and mitomycin C (MMC) were diluted in culture medium before each experiment. The treatment scheme and

Abbreviations: AS-ODN, antisense oligonucleotide(s); BCa, bladder cancer; CDDP, *cis*-diaminedichloroplatinum (cisplatin); CT, chemotherapy; hTERT, human telomerase reverse transcriptase; LF, Lipofectin; MMC, mitomycin C; NS, nonsense; qPCR, quantitative polymerase chain reaction; siRNA, small interfering RNA.

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TABLE I – SEQUENCES OF NUCLEIC ACID CONSTRUCTS. ALL ODNS CONTAINED TWO PHOSPHOROTHIOATES ON THE TERMINAL NUCLEOTIDES OF THE 5'-SITE AND THE 3'-SITE. SCRAMBLED NUCLEOTIDES OF THE SCR-ODNS IN COMPARISON TO AST2331 ARE DEPICTED UNDERLINED

Target	ODNs		siRNA target sequences	
hTERT	ASt2206[11]	TGTCCTGGGGGATGGTGTCG	si-hTERT1	CUGGAGCAAGUUGCAAAGCAU
	ASt2331[11]	GGTAGAGACGTGGCTCTTGA	si-hTERT2	CAGCUCCCAUUUCAUCAGCAA
–	SCR2	GGAAGTGAGGTCGCACTTGT		
	SCR3	GGTAGAGACGTCGCACTTGT		
	SCR4	GGAAGTGAGGTGGCTCTTGA		
	SCR5	GGTAGTGAGGTCGCACTTGA		
Survivin	AS-SVV[16]	AAGCGCAACCGGACGAATGC	si-SVV[17]	AAGCAUUCGUCCGGUUGCGCU
VEGF	AS-VEGF[18]	AGGGACCGTGCTGGGTCACC	si-VEGF	AGCACGGUCCCUCUUGGAAUU
–	NS-ODN[19]	TAAGCTGTTCTATGTGTT	NS-si	AAUUCUCCGAACGUGUCACGU

the analysis of apoptosis by annexin V staining were adapted from antisense experiments described earlier.[15]

Sample preparation for microarray hybridization

Total RNA was isolated using the RNeasy Mini Kit (Qiagen). After quality control by agarose gel electrophoresis, single stranded cDNA was prepared from 8 µg RNA template, using a T7-OligodT24-primer (TibMolbiol, Berlin, Germany) and Super-Script II (Invitrogen). Secondary strand synthesis of the cDNA was performed using *E.coli* DNA Ligase, *E.coli* DNA Polymerase I, RNase H and dNTPs (all from Invitrogen). The double stranded cDNA was purified by the GFX PCR DNA and Gel Purification Kit (Amersham Biosciences, Freiburg, Germany) and completely copied into biotin-labeled cRNA, using the Megascript T7 kit (Ambion, Woodward). The cRNA was purified using RNeasy columns (Qiagen) followed by quantification, fragmentation and hybridization on HG-U133 A arrays (Affymetrix, Santa Clara) for 16 hr at 45°C according to the users manual. The chips were scanned using a GeneArray scanner (Agilent, Palo Alto).

Data analysis and comparison strategies

All probe sets from each array were normalized to a target intensity of 500. The control probe sets (spiking controls, house keeping genes) were verified for absence of anomalies. Output files were analyzed by the dCHIP 1.3 software (www.dchip.org), using the PM-only model. The different arrays were normalized to the array with the median overall intensity, followed by calculation of model-based expression values and outlier detection. Combined comparisons were performed to identify differentially expressed genes in cells treated with hTERT AS-ODNs or siRNAs in comparison to the appropriate controls (NS-ODN, NS-si). In the first step, each AS-ODN array was compared to the NS-ODN array, and each siRNA array was compared to the NS-si array to exclude unspecific effects caused by the treatment regime (Fig. 1). The fold change used as cut-off was 2.0 for AS-ODN arrays. Because of the lower level in overall fold changes after siRNA treatment, the cut-off was set to 1.7 for siRNA arrays.

This procedure resulted in one specific gene list per AS-ODN or siRNA, respectively. The lists for the hTERT AS-ODNs or siRNAs were compared to those for constructs directed at survivin (AS-SVV, si-SVV) or VEGF (AS-VEGF, si-VEGF) to check for numbers of genes similarly regulated by the different treatments. The second step was the generation of 2 lists of genes containing those candidates, which were differentially expressed after antihTERT treatment with both of the AS-ODNs (ASt2206, ASt2331) or both of the siRNAs (si-hTERT1, si-hTERT2), each in comparison to the appropriate NS control. In a third step, these hTERT gene lists were compared to the survivin and VEGF arrays. Furthermore, the hTERT AS-ODN specific gene list and the hTERT siRNA specific gene list were compared (Fig. 1). The analyses employing NS-ODN, NS-si and si-hTERT2 were performed in 2 independent experiments, and the mean values were used for analysis.

Quantitative polymerase chain reaction (qPCR) analyses

The hTERT mRNA expression was quantified by the LightCycler TeloTAGGG hTERT Quantification Kit (Roche). The Super-

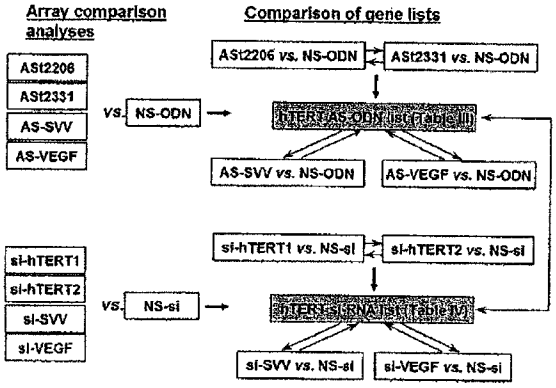

FIGURE 1 – Schematic description of the comparative analysis of mRNA expression patterns by oligonucleotide microarrays. The cells were treated with AS-ODNs targeted at hTERT (ASt2206 and ASt2331) and control AS-ODNs (AS-SVV and AS-VEGF) as well as with hTERT siRNAs (si-hTERT1 and si-hTERT2) and control siRNAs (si-SVV and si-VEGF). AS-ODN and siRNA arrays were normalized to NS-ODN and NS-si arrays, respectively.

script II reverse trancriptase (Invitrogen) and random hexamer primers (Amersham Biosciences) were used for the reverse transcription of 1 µg total RNA into first strand cDNA. All cDNAs were diluted 1:5 before performing the qPCR reactions. The alterations of the expression of selected genes (ATF3, EGR1, RHOB, PDCD4, RAB31, ID2) were confirmed by TaqMan Gene Expression assays (Applied Biosystems, Foster City) on the LightCycler instrument (Roche). For this purpose, serially diluted PCR fragments (10^2–10^6) were used to generate calibration curves. The primers and probes for the amplification of EGFR and PBDG are shown in Table II. The PCR for the reference gene TBP was adopted from Linja *et al.*[20] Each qPCR, except that of hTERT, was performed using the LC FastStart Master Hybridization Probe kit (Roche). The data represent means of independent duplicates.

EGFR protein detection by Western blotting

Western Blot analyses were performed according to a standard protocol, using monoclonal antibodies against EGFR (clone H9B4; 1:1,000) (Biosource, Solingen, Germany) and β-actin (1:8,000) (Sigma, St. Louis, MO). A secondary antimouse-HRP antibody (1:1,000) (Dako, Glostrup, Denmark) and the Enhanced Chemiluminescence Kit (Amersham Biosciences) were used for visualization.

Results

Effects of hTERT AS-ODNs on target expression

It was shown previously by us, that the AS-ODN-mediated reduction of the hTERT mRNA reached its maximum 12 hr after transfection.[11] Hence, this point of time was chosen to perform the

TABLE II – PRIMERS AND PROBES FOR THE QPCR OF THE EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) AND OF THE PORPHOBILINOGEN DEAMINASE (PBGD)

Gene (Fragment length)	Primer/probe	Sequence 5′-3′
EGFR (182 bp)	EGFR up	GGAATTGTTGCTGGTTGCACT
	EGFR down	AAAGAATGCATTTGCCAAGTCCTA
	EGFR FL	ATGAGGTACTCGTCGGCATCCACC–FL
	EGFR LC	LC–CGTCGTCCATGTCTTCTTCATCCATC–PH
PBGD (158 bp)	PBGD up	GCTGCAACGGCGGAA
	PBGD down	CCTGTGGTGGACATAGCAATGATT
	PBGD FL	TCGCATACAGACGGACAGTGTGGTG–FL
	PBGD LC	LC–CAACATTGAAAGCCTCGTACCCTGG–PH

FL, fluorescein; LC, LightCycler Red 640; PH, phosphate group.

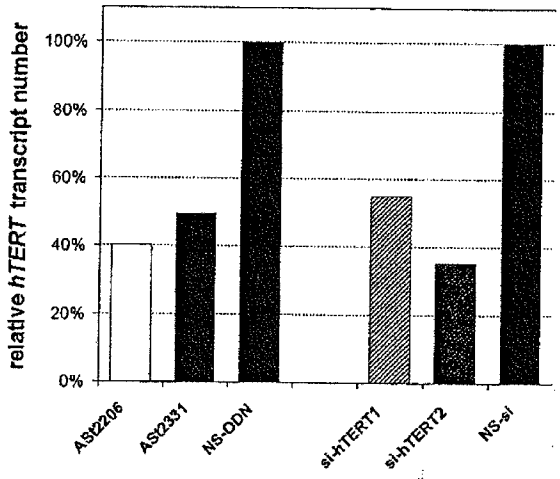

FIGURE 2 – Target-specific actions of AS-ODNs and siRNAs. The relative hTERT mRNA expression (hTERT/PBGD) was measured by qPCR at 12 hr (AS-ODNs) and 24 hr (siRNAs) after transfection. The relative hTERT transcript numbers were normalized to those of NS-ODN and NS-si treated samples, respectively.

microarray experiments. The efficient knock-down of hTERT was confirmed by means of qPCR in EJ28 cells (Fig. 2). Both of the hTERT AS-ODNs, ASt2206 and ASt2331 diminished the expression of their target 12 hr after transfection. ASt2206 reduced the hTERT mRNA level slightly more efficiently (to 40% of the NS-ODN) than ASt2331 (to 50%).

Expression profiling by oligonucleotide microarrays after treatment with hTERT AS-ODNs

Paired comparison analyses were performed to investigate influences on gene expression caused by both of the 2 hTERT AS-ODNs, but not by the NS-ODN (ASt2206 *vs.* NS-ODN, ASt2331 *vs.* NS-ODN) (Fig. 1). These effects—in the case of target specificity—should not appear after treatment with AS-ODNs targeted at survivin (AS-SVV) or VEGF (AS-VEGF), which served as controls.

The 2 hTERT AS-ODNs caused total numbers of changed genes of 59 (ASt2206) and 101 (ASt2331), respectively, whereby most of them were upregulated (Fig. 3*a*). Fifteen of a total of 59 genes (25%) changed by ASt2206 treatment were also affected by AS-SVV, whereas 25 genes (42%) were affected equally by AS-VEGF. The highest degree of concordance was revealed between ASt2331 and AS-VEGF (75%).

Furthermore, a list of genes changed in parallel by both of the hTERT-directed constructs ASt2206 and ASt2331 was generated (Table III). The comparison of this hTERT AS-ODN gene list with genes, whose expression was altered by AS-SVV and AS-VEGF, yielded a high degree of concordance: from the 28 genes within the hTERT gene list, 22 genes (79%) were also upregulated by AS-VEGF, 13 genes (46%) were coaffected by AS-SVV and 11 genes (39%) were altered together by all AS-ODNs independent of their target. Four genes were differentially expressed exclusively after treatment with hTERT AS-ODNs, but neither after treatment with AS-SVV nor with AS-VEGF. The sterol-C4-methyl oxidase-like *SC4MOL* and *FZD2* (frizzled homolog 2) were down-regulated. The hypoxia and DNA-damage-inducible transcript *DDIT4* and the hypothetical protein FLJ20707 were upregulated. Unexpectedly, a variety of genes known to be involved in stress response were induced by treatment with AS-ODNs against different targets: *IL6*, *IL8*, *EGR1*, *ATF3*, *CEBPB*, *GADD45*, *GADD34* and *MAFF* (Table III).

Taken together, the genes identified here were differentially expressed after treatment with AS-ODNs in comparison to the NS-control. The high degree of concordance between the different treatment groups leads to the assumption that these effects may be widely independent of the effects on the target of each of the AS-ODNs.

Effects of various AS-ODNs on cell growth

The 2 hTERT AS-ODNs efficiently reduced the numbers of EJ28 cells within the first 24 hr after transfection in comparison to the NS-ODN, whereby ASt2331 caused the more pronounced reduction to about 33% of the NS-ODN (Fig. 4). AS-VEGF was similarly efficient like ASt2331 at 24 hr, followed by AS-SVV.

Influence of scrambled hTERT AS-ODNs on cellular viability and target expression

Because of the high percentage of genes altered together by AS-ODNs directed at different targets, 4 additional control ODNs (SCR2, SCR3, SCR4, SCR5)—derived from ASt2331 with the most potent action on cellular growth—were analyzed to investigate the relationship between sequence and action of ASt2331. The scrambled SCR-ODNs containing 3–6 exchanged bases compared to ASt2331 (Table I) and thus without ability for hybridization to the hTERT mRNA were used to investigate their influence on viability and hTERT expression. Matching their sequences with the human genome database (http://www.ncbi.nlm.nih.gov/blast) yielded no significant homologies to known human mRNAs.

The scrambled SCR2 construct, modified by 6 base exchanges over the whole sequence, had neither an influence on cellular viability nor on hTERT expression (Fig. 5). SCR3 containing 3 base exchanges on the 3′-site reduced both the viability and the hTERT expression of EJ28 cells more efficiently than the primary ASt2331. A changed 5′-sequence (SCR4) or a changed central sequence (SCR5) had no or little effect on viability, whereby SCR5 caused a moderate hTERT repression.

The reduction of viability and hTERT expression by the modified version of ASt2331 (SCR3) with disordered homology to the hTERT mRNA let us assume, that—beside the effects on target expression—target-independent effects of the hTERT AS-ODNs contributed to the growth inhibition of BCa cells.

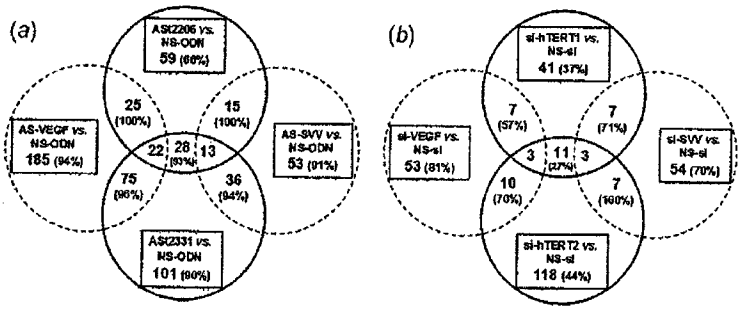

FIGURE 3 – Venn diagrams illustrating the numbers of differentially expressed genes and their overlaps. (*a*) Twelve hours after treatment with hTERT AS-ODNs. (*b*) Twenty-four hours after treatment with hTERT siRNAs. Each circle described the effect of a construct targeted at hTERT (solid line) or of a control construct (dashed line) directed at survivin (AS-SVV and si-SVV) or at the vascular endothelial growth factor (AS-VEGF and si-VEGF), each normalized to the NS-ODN or NS-si, respectively. Percentages of upregulated genes are shown in brackets.

TABLE III – hTERT AS-ODN GENE LIST. SHOWN ARE GENES, REGULATED TOGETHER BY BOTH OF THE hTERT AS-ODNs AND THE INFLUENCE OF AS-SVV AND AS-VEGF ON THE EXPRESSION OF THESE GENES. NEGATIVE FOLD CHANGES INDICATE DOWN-REGULATION

Probe set	Gene name	Symbol	Fold change				Function
			ASt2206	ASt2331	AS-SVV	AS-VEGF	
209146_at	Sterol-C4-methyl oxidase-like	*SC4MOL*	−2.3	−2.3	nc	nc	Cholesterol biosynthesis
210220_at	Frizzled homolog 2 (Drosophila)	*FZD2*	−2.0	−2.0	nc	nc	G-protein coupled receptor activity
202887_s_at	HIF-1 responsive RTP801	*DDIT4*	2.4	3.3	nc	nc	DNA damage response
220369_at	Hypothetical protein FLJ20707	–	2.1	2.8	nc	nc	Not known
36711_at	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	*MAFF*	5.0	3.0	nc	6.9	Transcription factor; cellular stress response
202912_at	Adrenomedullin	*ADM*	2.8	4.0	nc	4.4	Cell–cell signaling
202644_s_at	Tumor necrosis factor, α-induced protein 3	*TNFAIP3*	4.7	2.6	nc	4.0	Transcription factor; TNF-mediated apoptosis
205207_at	Interleukin 6 (interferon, β 2)	*IL6*	4.0	2.4	nc	2.9	Cell–cell signaling
205047_s_at	Asparagine synthetase	*ASNS*	2.7	2.4	nc	2.6	Metabolism
209270_at	Laminin, β 3	*LAMB3*	2.5	2.2	nc	2.6	Basement membrane protein
209305_s_at	Growth arrest and DNA-damage-inducible, β	*GADD45B*	2.4	2.1	nc	2.2	Apoptosis; cell cycle; MAPK pathway
201739_at	Serum/glucocorticoid-regulated kinase	*SGK*	2.1	2.4	nc	2.8	Response to stress
222162_s_at	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1	*ADAMTS1*	2.4	2.6	nc	3.0	Extracellular matrix degrading enzyme
209020_at	Chromosome 20 open reading frame 111	*C20orf111*	2.1	2.0	nc	2.3	Not known
220046_s_at	Cyclin L ania 6	*CCNL1*	2.7	2.7	nc	2.9	PremRNA processing
212501_at	CCAAT/enhancer-binding protein (C/EBP), β	*CEBPB*	2.2	2.8	2.6	nc	Transcription factor
217988_at	Cyclin B1 interacting protein 1	*CCNB1IP1*	2.3	3.2	2.6	nc	Cell cycle progression
202672_s_at	Activating transcription factor 3	*ATF3*	10.7	4.9	2.8	8.5	Transcription factor
201694_s_at	Early growth response 1	*EGR1*	3.7	2.8	3.6	4.0	Transcription factor
207768_at	Early growth response 4	*EGR4*	3.7	2.7	5.0	5.8	Transcription factor
202859_x_at	Interleukin 8	*IL8*	6.5	2.4	2.1	2.7	Cell–cell signaling
202014_at	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	*GADD34*	2.8	3.2	2.1	3.6	Stress response; apoptosis; cell cycle; DNA damage response
214062_x_at	Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, β	*NFKBIB*	2.1	2.1	2.1	2.6	Apoptosis
203439_s_at	Stanniocalcin 2	*STC2*	2.3	2.7	2.1	2.8	Cell–cell signaling
221577_x_at	Growth differentiation factor 15	*GDF15*	2.0	3.3	2.8	3.2	Bone morphogenetic protein; member of the TGFB1 family
220755_s_at	Chromosome 6 open reading frame 48	*C6orf48*	2.6	3.4	2.9	3.3	Not known
218750_at	Hypothetical protein MGC5306	*MGC5306*	4.0	3.0	2.0	3.6	Not known
213649_at	Homo sapiens cDNA FLJ36807 fis, clone ASTRO2000141	–	2.1	2.9	2.3	4.4	Not known

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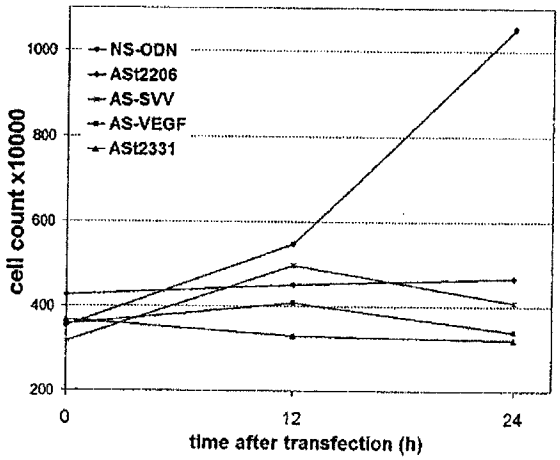

FIGURE 4 – Effects of transfection with hTERT AS-ODNs on EJ28 cell count at different time points after transfection.

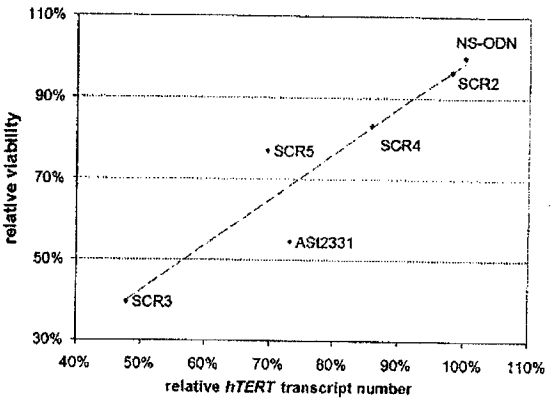

FIGURE 5 – Correlation between viability and hTERT expression after treatment with the hTERT targeting AS-ODN ASt2331 and its modified counterparts. The viabilities and the hTERT/PBGD expression ratios were normalized to that of the NS-ODN-treated samples.

Effects of hTERT siRNAs on target expression of BCa cells

The hTERT AS-ODNs were able to potently reduce the cellular viability as well as the expression of their target. Nevertheless, only 4 genes (without known association to telomerase function) were affected similarly by both of the hTERT AS-ODNs. Unexpectedly, a high number of genes were changed together after treatment with AS-ODNs directed at different targets. As an alternative method to inhibit hTERT, 2 different siRNAs (Table I) were used.

The most prominent reduction of the hTERT mRNA expression was seen at 24 hr after transfection (data not shown). The cells were harvested for microarray experiments at this point of time. The hTERT expression in EJ28 cells was inhibited by si-hTERT1 to 55% and si-hTERT2 to 35% in comparison to NS-si 24 hr after transfection (Fig. 2).

Expression profiling by oligonucleotide microarrays after treatment with hTERT siRNAs

Using the same procedure as described earlier (Fig. 1), hTERT was silenced by si-hTERT1 and si-hTERT2. The expression changes were analyzed in comparison to treatment with NS-si (without homology to any human mRNA) and compared to those obtained after transfection with si-SVV and si-VEGF, served as controls to differentiate between effects exclusively caused by the repression of hTERT or caused by a general siRNA-mediated mechanism independent of the target.

The total number of altered genes clearly differed between si-hTERT1 (41 genes) and si-hTERT2 (118 genes) (Fig. 3*b*). The proportions of upregulated genes were 37% after si-hTERT1 treatment and 44% caused by si-hTERT2. The degree of concordance between the different treatment groups was relatively low. Seven (17%) concordant genes were identified by comparing si-hTERT1 with si-SVV as well as by comparing si-hTERT1 with si-VEGF. Of these, 3 genes (*EREG*, *IL13RA2*, *RIG*) were found in both of the comparisons (data not shown). Ten of 118 (8%) and 7 of 118 genes (6%) were regulated in parallel by si-hTERT2/si-VEGF and by si-hTERT2/si-SVV, respectively. Of all these overlaps, F-box and leucine-rich repeat protein 11 (FBXL11) and Ras-GTPase activating protein SH3 domain-binding protein 2 (G3BP2) were identified as being altered by all siRNAs independent of their target (Table IV). T4

Eleven genes were found as regulated together by si-hTERT1 and si-hTERT2, whereof 7 genes (64%) were exclusively altered by the hTERT si-RNAs and neither by si-SVV nor by si-VEGF. All these 7 genes were down-regulated (Table IV). This hTERT siRNA gene list included 2 genes with known function as oncogenes: *EGFR* and *FOSL1* (also known as *FRA1*), which is a component of the AP-1 transcription factor complex. Furthermore, the epithelium-specific expressed laminin γ2 (*LAMC2*) gene was also specifically down-regulated by both si-hTERT1 and si-hTERT2.

Validation of the microarray data by quantitative PCR (qPCR) and Western blotting

qPCRs for 7 genes with high, moderate or low fold changes on microarrays were performed to verify the data. The fold changes measured by qPCR correlated well with those obtained by microarrays, whereby the qPCR showed a higher sensitivity (Table V). T5

Moreover, we investigated whether a reduction of the EGFR mRNA resulted in a reduced protein level in EJ28 cells by Western Blot analysis. The transfection with both si-hTERT1 and si-hTERT2 clearly diminished the EGFR protein after 24 hr in EJ28 cells, whereas si-VEGF (Fig. 6) and si-SVV (data not shown) had no effect on EGFR protein content. Similarly to the microarray and qPCR results, si-hTERT2 is more efficient than si-hTERT1, not only in inhibiting hTERT but also in down-regulating EGFR. F6

Effects of hTERT siRNAs in combination with chemotherapeutics on growth of EJ28 cells

No significant changes in viability, proliferation or apoptosis were observed after treatment with si-hTERT1 or si-hTERT2 24 hr after transfection (data not shown). However, a decreased number of cells entering the S-phase of the cell cycle 48 hr after the treatment with si-hTERT2 in comparison to the NS-si control (21 *vs.* 31%) was detected, whereas the population in G1 was increased (70 *vs.* 59%) (data not shown).

We have shown previously the enhancement of chemotherapy (CT)-mediated effects on cellular viability by pretreatment with hTERT AS-ODNs in several BCa cell lines.[15] The fact that AS-ODNs and siRNAs targeted at hTERT caused completely different changes on mRNA expression patterns of BCa cells raised the question whether hTERT siRNAs can also sensitize BCa cells to CT.

Preliminary data revealed that hTERT inhibition by si-hTERT2 followed by incubation with a relatively low concentration of MMC decreased the cell count of EJ28 cells in comparison to treatment with si-hTERT2 or MMC as single agents. A reduction in cell number by si-hTERT2 + MMC to 50% of the control, treated with NS-si + MMC, was noticed after 72 hr. The same effect was seen using CDDP. Moreover, the rate of apoptosis,

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TABLE IV – hTERT siRNA GENE LIST. SHOWN ARE GENES, REGULATED TOGETHER BY BOTH OF THE hTERT siRNAs AND THE INFLUENCE OF si-SVV AND si-VEGF ON THE EXPRESSION OF THESE GENES. NEGATIVE FOLD CHANGES INDICATE DOWN-REGULATION

Probe set	Gene name	Symbol	Fold change				Function
			si-hTERT1	si-hTERT2	si-SVV	si-VEGF	
201983_s_at	Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	*EGFR*	−1.9	−1.9	nc	nc	Receptor tyrosine kinase
204420_at	FOS-like antigen 1	*FOSL1*	−1.9	−1.8	nc	nc	Member of the AP-1 transcription factor complex
202267_at	Laminin, γ 2	*LAMC2*	−1.8	−1.9	nc	nc	Basement membrane protein; cell adhesion/ migration/differentiation
222162_s_at	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1	*ADAMTS1*	−1.7	−2.2	nc	nc	Extracellular matrix degrading enzyme
212501_at	CCAAT/enhancer binding protein (C/EBP), β	*CEBPB*	−1.7	−1.7	nc	nc	Transcription factor, interleukin 6-dependent DNA-binding protein
209675_s_at	Heterogeneous nuclear ribonucleoprotein U-like 1	*HNRPUL1*	−1.7	−2.2	nc	nc	Member of the hnRNP family
211996_s_at	KIAA0220-like protein	*LOC23117*	−1.8	−1.7	nc	nc	Not known
212406_s_at	Myelin transcription factor 1	*MYT1*	1.9	1.9	1.8	nc	Transcription factor
221577_x_at	Growth differentiation factor 15	*GDF15*	−1.8	−2.5	nc	−2.8	Bone morphogenetic protein; member of the TGFB1 superfamily
208841_s_at	Ras-GTPase activating protein SH3 domain-binding protein 2	*G3BP2*	1.9	1.7	2.1	1.8	Not known
208988_at	F-box and leucine-rich repeat protein 11	*FBXL11*	1.9	1.9	1.9	1.9	Member of the F-box protein family

nc, not changed.

TABLE V – VALIDATION OF DIFFERENTIALLY EXPRESSED GENES IDENTIFIED BY MICROARRAYS USING qPCR. THE EXPRESSION VALUES OF ATF3 AND EGR1 WERE NORMALIZED TO THE REFERENCE GENE *PBGD*; ALL OTHER EXPRESSION VALUES WERE NORMALIZED TO TBP. NEGATIVE FOLD CHANGES INDICATE DOWN-REGULATION

Gene	Treatment	FC microarray	FC qPCR
ATF3	ASt2206 *vs.* NS-ODN	10.8	43.9
	ASt2331 *vs.* NS-ODN	4.9	16.2
EGR1	ASt2206 *vs.* NS-ODN	3.7	12.4
	ASt2331 *vs.* NS-ODN	2.8	7.6
PDCD4	si-hTERT2 *vs.* NS-si	1.6	1.9
ID2	si-hTERT2 *vs.* NS-si	1.3	1.8
RHOB	si-hTERT2 *vs.* NS-si	−2.6	−4.9
EGFR	si-hTERT1 *vs.* NS-si	−1.9	−1.6
	si-hTERT2 *vs.* NS-si	−1.9	−2.6
RAB31	si-hTERT2 *vs.* NS-si	−1.5	−1.8

FC, fold change.

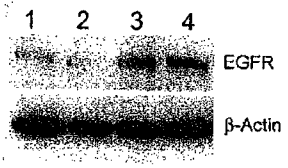

FIGURE 6 – Detection of EGFR protein content by Western Blotting. The lanes represent samples, transfected with si-hTERT1, si-hTERT2, si-VEGF3 and NS-si.4 The EJ28 cells (5×10^4 per sample) were harvested 24 hr after transfection, and protein lysates were separated on a 7.5% SDS-polyacrylamide gel. β-actin served as a control for equal loading.

measured as annexin V-positive cells, was specifically increased after si-hTERT2 + MMC treatment (33.7%) in comparison to NS-si + MMC (17.3%). A similar enhancement effect on apoptosis was shown for CDDP at 48 hr.

Discussion

Assessment of AS-ODN-mediated effects

The transfection with AS-ODNs targeted at hTERT led, on the one hand, to an efficient reduction of the hTERT mRNA but, on the other hand, to a high degree of concordance between genes changed by antihTERT treatment and those changed by control AS-ODNs against other targets. Possible effects caused simply by transfection with nucleic acids were excluded by normalization to NS-ODN-treated cells. Thus, the different AS-ODNs seem to act in an AS-ODN-specific rather than in a target-specific way. The possibility that genes were regulated in parallel by inhibiting hTERT, survivin and VEGF in a target-specific manner was not considered but cannot be excluded in the results shown here.

Several genes present in the hTERT AS-ODN gene list (Table III) are described to be connected at a regulatory level. Many of them are associated with response to stress, e.g. *EGR1*, an early growth response gene, inducible by genotoxic and mitotic stress in normal and neoplastic cells[21] as well as by genistein treatment in BCa cells.[22] EGR1 is described as a tumor-suppressor, implicated in the regulation of cell growth and transformation and shown to positively regulate the expression of growth suppressive genes such as *GADD45*[23] and *GDF15*,[24] which were also upregulated by different AS-ODNs in the present study. GDF15 (also known as NAG-1) has an antitumorigenic function in human colorectal carcinoma cells.[24] EGR1 was shown to be simultaneously upregulated together with the transcription factors ATF3 and CEBPB after acute pancreatitis.[25] Besides inflammation processes, endoplasmatic reticulum stress induced the expression of ATF3 and CEBPB.[26,27] ATF3 in turn can induce the expression of GADD34 in mouse embryo fibroblasts.[28] After initiation of genotoxic stress, CEBPB is implicated in the transcriptional control of DDIT4 (also known as RTP801), a mediator in the mitochondrial apoptotic

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pathway, which was able to impair cell growth by inhibiting the stress-regulated mTOR-signaling pathway.[29]

Benimetskaya *et al.* analyzed the gene expression of prostate cancer cells after transfection with G3139, an AS-ODN targeting BCL2, and found target-independent expression profiles related to those obtained after interferon treatment.[30] In accordance with our data, nearly all of the candidates were upregulated, and 5 of these genes were also induced by the hTERT AS-ODNs in the present study (*ATF3*, *ADM*, *GADD45B*, *MAFF* and *IL8*).

In conclusion, the treatment with AS-ODNs targeting different tumor-associated genes caused the induction of a complex network of growth inhibiting factors, which are related to a response to stress, whereby EGR1 seems to be a major regulator of this process. The detailed cause of this stress remains to be clarified. However, the reduced hTERT expression may be cause or/and consequence of the induced cell death. For the selection of reliable AS-ODNs, we recommend the comparative analysis of suitable control molecules, for instance at least one additional AS-ODN against another target as well as a NS-ODN.

The microarray results implicated a nontarget specific way of action of the investigated hTERT AS-ODNs, which was described in a similar manner for the BCL2-directed AS-ODN G3139, whose function in PC3 prostate cancer cells depends on a "bis-CpG" (*CGTGC*) motif.[31] Interestingly, the hTERT AS-ODN ASt2331 contained an analogous sequence motif (CGTGGC) (Table I). The investigation of additional control ODNs (SCR2–SCR5), with base substitutions in comparison to the parental ASt2331 and thus without the ability to stably bind to the hTERT mRNA, revealed the complete loss of function of SCR2. In this construct, the "bis-CpG" motif was disrupted. On the other hand, base substitutions within SCR3, which had an effect on cell growth and hTERT expression, kept the 2 CpG motifs unaltered (*CGTCGC*). Alternatively, the retained function of SCR3 could be explained by an unchanged 5′-site, because 3 base exchanges were introduced only at the 3′-site of the construct. However, the "bis-CpG" hypothesis of action cannot be transferred to the other hTERT targeting AS-ODN ASt2206. It contained no CpG motif but a G-quadruplex sequence, known to cause antiproliferative effects *in vitro*.[32] The appearance of 2 different known sequence motifs with growth suppressing function and the low overlap of gene signatures between ASt2206 and ASt2331 could refer to diverse modes of action of these AS-ODNs.

To exclude the possibility that the effects of the hTERT AS-ODNs and SCR constructs are a phenomenon of EJ28 cells, the growth inhibition and target reduction were confirmed in 5637 BCa cells with similar results (data not shown).

Assessment of siRNA-mediated effects

The investigated hTERT AS-ODNs indeed reduced their target mRNA, but the effects on cell growth seemed to be associated with stress response rather than with specific telomerase down-regulation. Thus, hTERT was targeted by an independent technique, using 2 siRNAs and appropriate control siRNAs to yield a specific transcriptional response to the reduced hTERT level.

The higher efficacy of si-hTERT2 compared to si-hTERT1 to repress the target was confirmed using both another transfection reagent (DOTAP instead of LF) and another BCa cell line (5637) (data not shown). The different activity could explain the higher number of genes changed by si-hTERT2 treatment. On the other hand, differences in the behavior of distinct siRNAs against the same target regarding their effects on mRNA expression patterns could be explained by the toleration of one to several mismatches within the target sequence, leading to cross-reactions with untargeted genes of limited sequence homology.[33] Nevertheless, 11 genes were affected in parallel by both of the hTERT siRNAs.

The appearance of siRNA-mediated off-target effects was described by Persengiev *et al.*, who investigated the global gene expression after treatment with siRNAs targeted at the nonmammalian luciferase gene in HeLa cells and found a nonspecific signature of >1,000 genes.[34] The hTERT siRNA list (Table IV) contained no gene from this signature, being probably caused by cell-specific varieties and different comparison criteria. Persengiev *et al.* normalized the effects to untreated cells, whereas a NS-si construct was used for normalization in the present study. However, 2 candidates for off-target effects were identified as changed after transfection with different siRNAs independent of their target: *FBXL11* and *G3BP2*. The G3BP2 protein contains RNA-binding motifs and is implicated in RNA metabolism.

Semizarov *et al.* compared the expression profiles of human lung cancer cells after treatment with siRNAs targeted at AKT1, PLK1 or RB1.[35] The overlap of the individual expression patterns ranged from 8 to 38%. This is in accordance with the results presented here: 6–17% of genes were similarly regulated by siRNAs against different targets.

To our knowledge, the down-regulation of the oncogene *EGFR* by the siRNA-mediated inhibition of hTERT in BCa cells was reported for the first time in the present study. EGFR is described to be overexpressed in BCa tissues[36] and to facilitate motility and subsequent invasion of BCa cells.[37] Moreover, the overexpression of EGFR within the urothelium of transgenic mice in combination with the expression of SV40 large T antigen promotes the growth of BCa.[38] A putative connection between EGFR and telomerase was described previously in few reports, whereby it is not clear whether telomerase regulates EGFR or *vice versa*. Tian *et al.* showed a direct correlation between EGFR protein expression and telomerase activity as well as the down-regulation of telomerase activity in glioblastoma cells treated with EGFR AS-ODNs.[39] The reduction of telomerase activity in skin carcinoma cells treated with an EGFR-specific antibody or inhibitor, respectively, resulted from a diminished hTERT mRNA expression.[40] Furthermore, a direct induction of hTERT expression by EGF, the ligand of the EGFR, was described.[41] These reports support the hypothesis that EGFR may regulate the expression of hTERT. The other case, a possible regulation of EGFR by hTERT, was shown by the ectopic expression of hTERT in human mammary epithelial cells. The transfectants were characterized by a growth advantage caused by increased expression of growth promoting genes like *FGF2* and *EGFR*.[6] The results from the literature and our own data suggest mutual mechanisms of regulation of EGFR and hTERT.

Two microarray-based studies independently found an association of the expression of both *EGFR* and *FOSL1*, a member of the *FOS* proto-oncogene family, with the invasive phenotype of BCa cells and with the metastatic phenotype of human hepatocarcinoma cells.[42,43] The relevant function of FOSL1 in the development of epithelial tumors has been previously suggested.[44] A comparison of the gene signatures of tissue samples, derived from normal urothelium and from BCa, revealed a significant upregulation of FOSL1 in malignant tissues.[45] Furthermore, Macleod *et al.* showed an association between increased expression of FOSL1 and EGFR and the resistance against CDDP in ovarian cancer cells.[46] The decreased levels of FOSL1 and EGFR after siRNA-mediated hTERT inhibition in the present study could contribute to the sensitization of BCa cells to CDDP.

Another gene associated with invasive growth of tumor cells and selectively down-regulated after hTERT inhibition is laminin γ2 (LAMC2). It codes for the γ2 chain of laminin 5, a protein of the extracellular matrix. The importance of LAMC2 for tumor invasiveness was shown for several types of cancer, including BCa. Its overexpression significantly increased the risk of local tumor relapse of BCa patients.[32,47] Interestingly, 3 studies suggested a connection between LAMC2 expression, invasiveness of tumor cells and EGFR. LAMC2, which contains a laminin-type EGF-like domain, was not only induced by EGF,[48] but also coexpressed with EGFR in tumor cells.[49] Thus, EGFR may upregulate the expression of LAMC.

The comparison between the gene lists for hTERT AS-ODNs and hTERT siRNAs yielded no genes, which were regulated together in the same direction. Anderson *et al.* comparatively inves-

tigated the expression profiles of prostate cancer cells treated with AS-ODNs and siRNAs targeted at BCL2 by microarrays with similar results. Both AS-ODNs and siRNAs down-regulated the expression of BCL2, but the expression profiles showed nearly no overlaps.[50] In accordance with the present study, a target-independent AS-ODN-specific expression signature, which contained mainly stress-inducible genes, was identified.

Both hTERT-directed AS-ODNs and siRNAs reduced their target mRNA, but only AS-ODNs inhibited the growth of tumor cells immediately and potently. This might be associated with off-target effects of these inhibitors. Thus, the previously reported chemosensitization of BCa cells by hTERT AS-ODNs[15] might also be caused, at least in part, by off-target effects. In contrast, preliminary data showed the enhancement of the cytotoxic action of 2 CT by hTERT inhibition using siRNAs. This observation is in accordance with the previously described function of hTERT in the repair of CDDP-mediated DNA damages.[2] However, it remains to be evaluated in detail, whether a siRNA-mediated hTERT inhibition could lead to disturbed repair of DNA damages.

In conclusion, both of the nucleic acid-based inhibitors, AS-ODNs and siRNAs, affect not only their target gene but may cause various effects on the transcriptional level of other genes, whereby the expression patterns for hTERT siRNAs were more specific than that for hTERT AS-ODNs regarding their similarity to the controls. The hTERT AS-ODNs described here may mainly induce a stress response-like mRNA expression pattern, which causes the immediate and potent suppression of growth of BCa cells. The parallel repression of the hTERT mRNA may be a secondary effect, associated with the upregulation of several growth inhibiting genes. In contrast, we could show for the first time that a siRNA-mediated repression of the hTERT mRNA caused the down-regulation of the oncogene *EGFR*, known to be associated with telomerase. However, independent studies, particularly in different BCa cell lines, are needed to confirm the results presented herein and to clarify the function of hTERT as a putative inducer of growth promoting genes.

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